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Lipid trafficking in polarized hepatocytes, relation to bile secretion and the biogenesis of the bile canalicular membrane

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Summary and concluding remarks

The objective of this thesis was to obtain insight in the intracellular sorting and transport of lipids in hepatic cells. Hepatocytes are polarized cells with well-defined basolateral (sinusoidal) and apical (bile canalicular) plasma membrane domains. The emphasis of the work was in particular on lipid trafficking in relation to the biogenesis of the bile canalicular membranes and the secretory pathways of lipids involved in the formation of bile. Extensive lipid trafficking occurs in the hepatocytes, in both the endocytic and exocytic, as well the biosynthetic pathway. In addition, the liver secretes a substantial amount of cholesterol and phospholipids into bile. The composition of biliary lipids is unique in that respect that it is distinctly different from the lipid composition of the bile canalicular membrane and other intracellular membranes. The specific lipid composition of the membranes and bile is most likely achieved by elaborate sorting and transport mechanisms.

In order to study the transport and sorting of lipids, markers are needed that allow monitoring the lipid flow. Radioactive lipids have proven very useful in whole organ and animal studies. However, detailed investigations on the level of individual cells calls for other markers. Fluorescent lipid analogs were used throughout the work described in this thesis. These are lipids with a fluorescent group attached to either the headgroup or the glycerol (phospholipids) or ceramide (sphingolipids) backbone via a carbon spacer at the position of a fatty acid chain.

In chapter 2 the hepatic processing of *N*-Rh-PE (*N*-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine), a headgroup labelled phosphatidylethanolamine analog, *in vivo* and *in vitro* is described. In rats with permanent heart and bile duct catheters, plasma clearance of small unilamellar vesicles containing *N*-Rh-PE and tracer amounts of [^3H -oleoyl]*N*-Rh-PE was monitored, and the biliary secretion of the rhodamine and ^3H label was determined. Approximately 50 % of the injected rhodamine label was retrieved in bile after 2 h time, while a much lower amount of the ^3H was found (6 %). The rhodamine label in bile was recovered as an until now unidentified chloroform soluble metabolite. The ^3H label was found mainly incorporated in bile acids. The data demonstrated that *N*-Rh-PE was rapidly cleared from the circulation, taken up by the hepatocytes, processed, targeted to the bile canaliculus and secreted. Further characterization of the intracellular transport pathways in the hepatocytes was done in a freshly isolated rat hepatocyte couplet system. *N*-Rh-PE was inserted into the plasma membrane at 4 °C, at which temperature endocytosis is blocked. Warming the cells to 37 °C resulted in rapid internalization. Fluorescence was initially seen in clusters of vesicles throughout the cells, and subsequently accumulated in pericanalicular vesicles and the bile canalicular lumen. The same metabolite as found in the bile duct cannulated rat experiments, was also detected in the hepatocyte couplets.

Details of the intrahepatic transport of *N*-Rh-PE were subsequently obtained in the isolated perfused rat liver system, employing in particular fractionation techniques (chapter 3). It was shown that infusion of *N*-Rh-PE was taken up by the liver, metabolized, transcytosed, concentrated and rapidly secreted into bile. The biliary secretion kinetics of rhodamine fluorescence after infusion of *N*-Rh-PE was compared with that of a well defined fluid phase marker, FITC-dextran. Identical secretion kinetics suggested that internalization and initial transport occurred via an endocytic

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pathway. The secretion of rhodamine fluorescence was effectively blocked at 16°C , indicating that vesicular transport through the endosomal compartment was likely involved. Evidence for the vesicular nature of this transport process was further derived from the observation that rhodamine fluorescence colocalized with fluorescein fluorescence in endosomal fractions. These vesicle fractions were obtained from subcellular liver fractions isolated from rats that were coinjected with N-Rh-PE and FITC-dextran, and purified by free flow electrophoresis. The efficient biliary processing of N-Rh-PE and its metabolites was not governed by its bulky headgroup, as secretion of rhodamine fluorescence was not hampered under conditions where the secretion of Rhodamine123, a structural analog of the headgroup was blocked.

Next, a set of experiments was developed to determine at the level of individual cells how lipids are transported from different intracellular sites towards bile or the bile canalicular membrane. For this purpose, rather than freshly isolated couplets, which were extremely sensitive to deterioration due to experimental manipulation, HepG2 cells were used as model system. This is a highly differentiated human hepatoma cell-line, that forms bile canaliculi in vitro. In this model system the biogenesis of the bile canalicular membrane as well as intracellular lipid trafficking pathways were studied (chapter 4). After insertion of N-Rh-PE in the basolateral plasma membrane it could be shown that internalization of the lipid analog resulted in the accumulation of rhodamine fluorescence in small intracellular vesicles and in large *intra-* and *intercellular* structures that were lined with microvilli (MLV, microvilli-lined vesicles). These MLV were identified as structures in the process of forming (intracellular) or representing (intercellular) bile canaliculi, based on morphological data and on the fact that they were recognized as apical target by N-Rh-PE. Co-labelling studies were performed with N-Rh-PE and a fluorescent ceramide analog, 6-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-ceramide ($\text{C}_6\text{-NBD-Cer}$). The latter analog is a sphingolipid precursor that accumulates in the Golgi apparatus at low temperatures and after raising the temperature is converted to fluorescent sphingomyelin (SM) and glucosylceramide (GlcCer) analogs. It was demonstrated that the MLV are interacting by vesicular mechanisms with both the basolateral membrane and with the Golgi compartment. The latter occurs in a direct, transcytosis-independent manner. This is a novel observation, because previous studies on the trafficking of apical membrane proteins have thusfar not revealed a transcytosis-independent pathway from the Golgi to the bile canaliculus in hepatocytes. In various epithelial cell lines, GPI-linked proteins are thought to be apically sorted in a direct manner. This possibly occurs via special (glyco)sphingolipid-rich microdomains, that originate from the Golgi complex, into which proteins are sorted *en route* to the apical plasma membrane.

It is yet to be excluded whether some GPI-linked proteins can be sorted and apically transported in hepatocytes in a direct manner as well.

To investigate a possible cotransport of certain proteins and sphingolipids in HepG2, and a potential functional relationship between lipids and proteins involved in transport and sorting, a photoaffinity labelled ceramide ($^{125}\text{I-N}_3\text{-Cer}$) was synthesized. In HepG2 cells, this probe was metabolized to mainly photoaffinity sphingomyelin. Chapter 5 describes the labelling of a limited number of proteins with affinity for ceramide. Of special interest were two proteins, one of 27 kD and one of 60 kD, which presumably interacted with Golgi-derived sphingomyelin. These proteins interact with sphingomyelin at (different) intracellular or apical location, the 60 kD protein being a membrane-bound protein. Additional experiments are underway to prove the potential

role of these proteins as chaperons in lipid trafficking between the Golgi and the apical membrane. Preliminary experiments have shown that cross-linking cellular proteins to ^{125}I -N₃-Cer or ^{125}I -N₃-SM has an inhibitory effect on trafficking of NBD-lipids from the Golgi to the apical membrane, but not to the basolateral membrane (chapter 6). Based on the observation that ceramide is not integrated in vesicular traffic to the MLV, we speculate that especially the two proteins with affinity for SM participate in direct Golgi to apical membrane transport.

The studies in chapter 6 were designed to further establish the characteristics of lipid trafficking to the apical membrane, both from the plasma membrane (the transcytotic route) and from the Golgi (the biosynthetic route). It was shown that *de novo* synthesized NBD-lipids are valid reporters of transcytosis-independent Golgi to MLV transport, because inhibition of sphingolipid synthesis by PDMP abolished the appearance of NBD-fluorescence in the MLV. It was further shown, using plasma membrane inserted *N*-Rh-PE as a marker for the transcytotic pathway and *de novo* synthesized NBD-lipids as transcytosis-independent Golgi to MLV transport markers, that transcytotic transport involved a microtubule dependent step, but did not rely on intact actin filaments. Direct Golgi to MLV transport however was relatively independent of microtubules, but perturbation of the actin filaments grossly altered the appearance of the Golgi apparatus and hampered the delivery of NBD-lipids to the MLV. It was subsequently shown that although exogenously administered C₆-NBD-SM and C₆-NBD-GlcCer both accumulated in the MLV, the sphingomyelin analog, in contrast to the glucosylceramide analog, was not confined to this compartment, and with increasing time, was retrieved from the MLV and relocated to the basolateral plasma membrane. Apart from the observation that the sphingomyelin analog did not seem confined to the MLV, it is also interesting to note that modification of lipids with a fluorescent tag as such did not automatically give rise to non-selective removal and secretion into bile.

In conclusion, the work described in this thesis resulted in the identification of two intracellular pathways involved in lipid flow toward the bile canalicular membrane: firstly, a transcytotic pathway, involving entry via the non-clathrin coated pathway, transfer to the endosomal compartment and vesicular transcytotic carriers, mediating the transport of lipid molecules. One or more steps in this pathway depend on microtubules, but not on actin filaments. Some lipids inserted in the apical membrane via this pathway can probably be retrieved and recycle. Secondly, a transcytotic independent pathway for Golgi-derived lipids, involving vesicular carriers and cellular proteins that may direct the flow of distinct lipids. Part of this pathway probably depends on actin filaments, but it is independent of microtubules. The two pathways are depicted in figure 1.

Now that some lipid trafficking pathways in polarized hepatocytes have been surveyed and (partly) characterized, it will be challenging to investigate the relation between lipids and proteins in these routes, and identify the role of lipids in protein sorting and proteins in lipid sorting. It is becoming increasingly clear that (small) proteins play a key function in the complex movement of vesicles through the cell, constituting an integral part of the secretory, transcytotic, and biosynthetic pathway as well as interorganellar communication. A functional relationship between lipids and specific proteins may govern the destination of membrane microdomains or complete vesicles, in line with analogous speculations as to the functional clusters of sphingolipids and glucosyl-phosphatidyl-inositol anchored proteins.

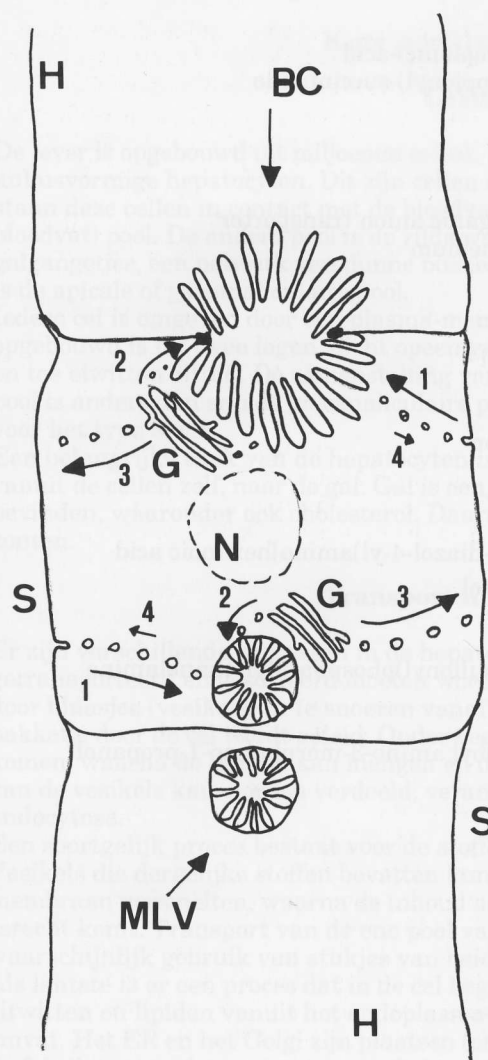


Figure 1: Lipid trafficking routes in polarized hepatocytes. The plasma membrane of polarized hepatocytes (H) consists of distinct sinusoidal (S) or basolateral domains, which are in contact with the blood circulation, and the bile canalicular (BC) or apical domain, where bile secretion occurs. The biogenesis of the mature, intercellular bile canaliculi, involves large intracellular microvilli-lined vesicles (MLV), which already intracellularly bear the morphological characteristics of bile canalicular membranes, and are recognized by apically targeted transport systems. Lipid trafficking routes identified thus far are (1): the transcytotic pathway from the basolateral to the apical membrane, involving non-(clathrin)-coated entry, the endosomal compartment, and vesicular carriers translocating lipids to the bile canaliculi. In this pathway, the transport steps beyond the endosomal compartment are sensitive to microtubule disrupting drugs, while it is relatively independent of intact actin filaments. (2): the transcytosis-independent Golgi to apical membrane transport route, which is probably dependent on actin filaments, but not on microtubules, and in which we propose a role for two sphingomyelin binding proteins. (3): the Golgi to basolateral membrane transport route, which is insensitive to microtubule

disrupting drugs, and does not rely on the previously mentioned sphingomyelin binding proteins. (4): possibly a transcytotic pathway from the apical to the basolateral membrane, which allows some lipids to be retrieved from the bile canalicular membrane and to recycle to the basolateral membrane. (N), nucleus; (G), Golgi apparatus.

Abbreviations

AIPP:	N-(3(azido-m-iodophenyl)propionic)-acid
AIPPS:	N-(3(azido-m-iodophenyl)propionyl)-succinimide
BHK:	baby hamster kidney
BSA:	bovine serum albumin
CD:	cytochalasine D
Cer:	ceramide
cMOAT:	canalicular multispecific organic anion transporter
DMEM:	Dulbecco's modified Eagle medium
FCS:	fetal calf serum
FFE:	free flow electrophoresis
FITC:	fluorescein isothiocyanate
GPI:	glycosylphosphatidylinositol
GTP:	guanosine 5'-triphosphate
GDP:	guanosine 5' diphosphate
HBSS:	Hanks balanced salt solution
IPRL:	isolated perfused rat liver
MDCK:	Madin-Darby canine kidney
MLV:	microvilli-lined vesicles
C ₆ -NBD:	6-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoic acid
GPI:	glucosyl-phosphatidyl-inositol
GlcCer:	glucosylceramide
MDR:	multi drug resistance
N-Rh-PE:	N-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine
PBS:	phosphate buffered saline
PC:	phosphatidylcholine
PDMP:	D,L-threo-1-phenyl-2-decanoyl amino-3-morpholino-1-propanol
PE:	phosphatidylethanolamine
PgP:	P-glycoprotein
PI:	phosphatidylinositol
PNS:	post nuclear supernatant
Rh:	rhodamine
SDS:	sodium dodecyl sulphate
SM:	sphingomyelin
TC:	taurocholate
TCDC:	taurochenodeoxycholate
TDHC:	taurodehydrocholate
TGN:	trans Golgi network
TLC:	thin layer chromatography
TUDC:	tauroursodeoxycholate